STUDIES ON THE ANTIMICROBIAL ACTIVITY OF N-SUBSTITUTED MALEIMIDES. II. FURTHER EXAMINATIONS ON N-PHENYLMALEIMIDE AND RELATED COMPOUNDS

L. FERENCZY*—B. MATKOVICS**—J. ZSOLT*
*Institute for Plant Physiology of the University, Szeged
**Institute for Organic Chemistry of the University, Szeged

Fungistatic activity of N-phenylmaleimide was demonstrated in a previous paper (FERENCZY—ZSOLT—MATKOVICS 1959). This paper deals with further investigations concerning N-phenylmaleimide and the antifungal activity of some N-substituted maleimides of different solubility.

Material and methods

The following derivates were investigated: N-phenylmaleimide (PMI), N-(o-oxyphenyl)-maleimide, N-(m-nitrophenyl)-maleimide, N-(p-acetyl)-maleimide, N-ethylmaleimide, N-α-naphthylmaleimide. Methods of synthesis are published elsewhere (MATKOVICS—FERENCZY—SELMECZI 1959).

Saccharomyces cerevisiae „GYŐR" (a Hungarian distillery yeast), *Actinomucor repens, Aspergillus foetidus, Aspergillus niger, Aspergillus oryzae, Circinella minor,* and *Syncephalastrum racemosum* were used as test organisms.

The nutrient solution employed for turbidimetrical investigations contained as follows: 5 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0,5 g $MgSO_4 . 7H_2O$, 10 g sucrose, and 1 ml yeast autolysate in 1 litre tap water. The maleimides and, in some cases, more yeast autolysate were added to this solution. To obtain yeast autolysate 10 g of fresh baker's yeast were suspended in 100 ml of tap water and kept at 65° C for 24 hours and then filtered. Sterilization and heat treatment, depending on the aims of examinations, was performed partly with Seitz EK filter, partly in 100° C water bath, and partly in autoclave at 0,5 atm overpressure for 15 minutes.

The nutrient solutions (10 ml in each tube) were inoculated with a drop of a suspension of the yeast *Saccharomyces cerevisiae* „GYŐR" harvested from a 3 days old malt agar slant. Each drop contained about 10^4 cells. Incubation was allowed at 25° C. The growth of yeast cultures was turbidimetrically measured in a photoelectric densitometer apparate (typ: Magnephot I., Orion, Budapest). For the diagramms the measured values were calculated into percents; the transparency of the uninoculated tubes was considered as 100 per cent.

Working with molds the activity of the aqueous and acetonic solutions of maleimides was measured with hole-plate agar diffusion test. Surfaces of plates (the media taken from the nutrient solution described above solidified with 2% agar) were inoculated with conidia of *Actinomucor repens, Aspergillus foetidus, Aspergillus niger, Aspergillus oryzae, Circinella minor,* and *Syncephalastrum racemosum* respectively. Holes of 10 mm diameter were made in the inoculated plates, 5 holes in each. The solutions of the active compound as well as the pure solvent were dropped into the holes. The plates were allowed to stand for 24 hours at 4° C and then incubated at 25° C. The zones of inhibition were measured after 36 hours. The standard error was $\pm$ 1,5 mm. The pure solvents did not give any inhibition. Each examination was performed in 5 parallels.

Results and discussion

Stability of PMI

Growth inhibiting activity of nutrient solutions containing PMI was considerably diminished when sterilized in autoclave. The growth curves of *Saccharomyces cerevisiae* „GYŐR" are shown in Fig. 1.

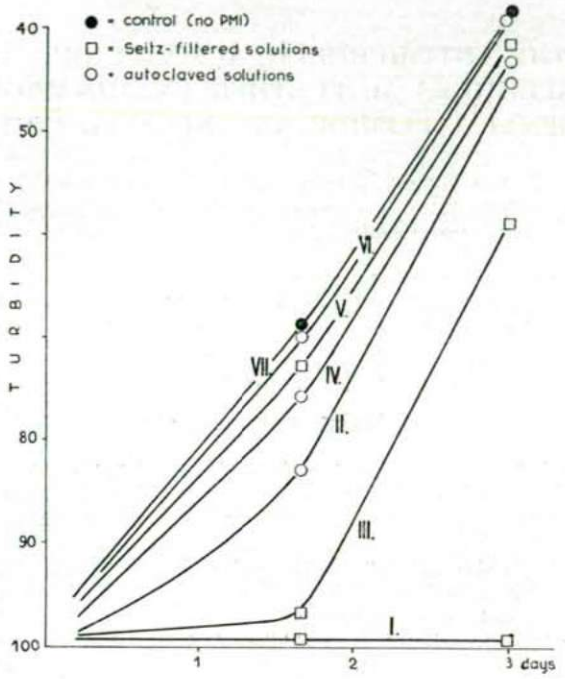

Fig. 1. Growth of *Saccharomyces cerevisiae* „GYŐR" in Seitz filtered and autoclaved nutrient solutions containing different amounts of PMI. I., II.=100 p. p. m. PMI. III., IV.= 10 p. p. m. PMI. V., VI.=1 p. p. m. PMI. VII.=control (without PMI).

Influence of pH was examined by autoclaving 10 p. p. m. of PMI in aqueous solutions at different pH. After autoclaving the solutions were neutralized and their activity was measured with agar diffusion test against *Saccharomyces cerevisiae* „GYŐR". Results are summarized in Table I. Accordingly, PMI is thermostabile at pH 2 and, on the other hand, it was destroyed at pH 9. Activity decreased considerably at pH values ordinarily used in microbiological media (Table I).

The yeast autolysate as a constituent of the nutrient solutions, influencing stability of PMI, has been demonstrated. Fig. 2. shows the results of experiments obtained with nutrient solutions containing 100 p. p. m. of PMI and different amounts of yeast autolysate. One set of the tubes was autoclaved, while in an other one the solutions were sterilized by Seitz-filtering. In all of

the solutions sterilized by Seitz-filtering independently of the amount of the yeast autolysate no growth was observed. In the autoclaved solutions the inhibition of growth was always decreased compared with the Seitz-filtered ones.

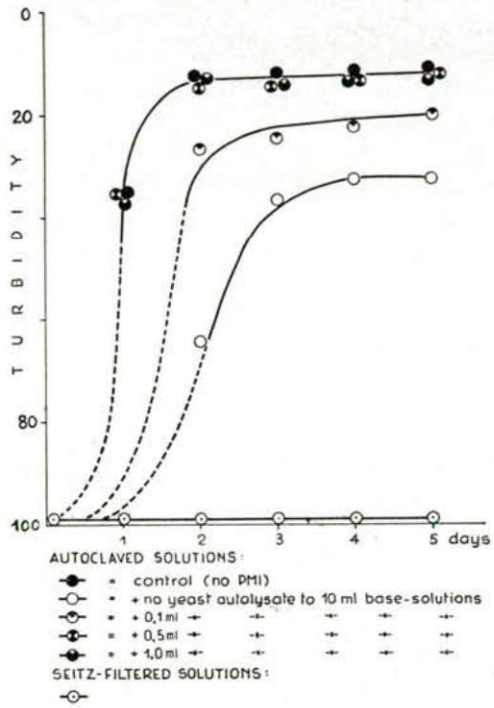

Fig. 2. Growth of *Saccharomyces cerevisiae* „GYÖR" in Seitz filtered and autoclaved nutrient solutions containing PMI in the presence of different amounts of yeast autolysate.

Decrease of the inhibitory activity was proportional to the amount of yeast autolysate added. The inhibitory activity entirely ceased in the autoclaved tubes containing 0,5 and 1,0 ml autolysate in 10 ml solution.

Influence of the duration of heating at 100° C was also investigated in nutrient solutions containing 100 p. p. m. of PMI and 1 ml yeast autolysate in 10 ml solution. Results are shown in Fig. 3. In solutions heated only for 5, 10, and 20 minutes the inhibitory activity of PMI was complete. However growth was only slightly inhibited in nutrient solutions heated for 40 and 60 minutes.

Activity of different N-substituted derivatives

Data obtained with solutions containing 10^{-3} M of PMI, N-(o-oxyphenyl)-maleimide, N-(m-nitro)-phenylmaleimide, N-(p-acetyl)-maleimide, N-ethylmaleimide, and N-α-naphtylmaleimide are summarized in Table II. These experiments were performed with the agar diffusion test against molds. As seen, *Aspergilli* are strongly inhibited by all the six compounds, while the other 3 molds remained unaffected (Table II).

The same selectivity in the inhibitory activity and the similar intensity of activity of the different compounds suggests a similar mechanism with all the six compounds.

The known effect of maleimides as auxin antagonists demonstrated by VELDSTRA (1946) and van OVERBEEK—BLONDEAU—HORNE (1955) render probable that the inhibitory action against fungi is connected with their possible reaction with sulfhydryl groups. This reaction may explain also the decrease of inhibitory activity of PMI by heating in the presence of yeast autolysate. The resistance of some species of molds to maleimides is still in need of further investigations.

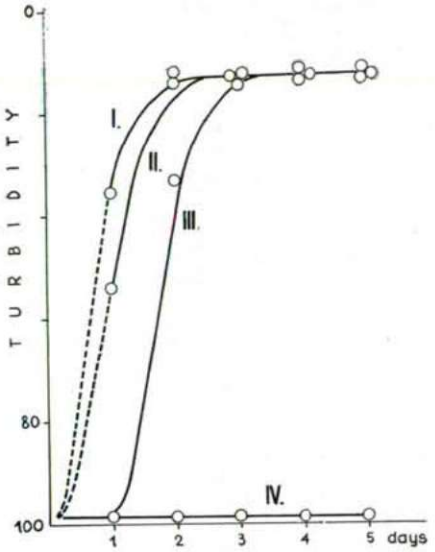

Fig. 3. Growth of *Saccharomyces cerevisiae* „GYŐR" in nutrient solutions containing 1 ml yeast autolysate per 10 ml solution and 100 p. p. m. PMI and heated for 60 (II.), 40 (III.), 20, 10 and 5 (IV.) minutes in a water bath of 100° C. I=control (without PMI).

Summary

The inhibitory activity of PMI in various circumstances was investigated against several fungi. PMI proved to be thermostabile at pH 2 but thermolabile at pH 4 to 9. The inhibitory activity of PMI is destroyed by autoclaving in nutrient solutions containing yeast autolysate. Boiling in 100° C water bath destroys to a certain extent the activity of PMI according to the duration of heating.

Six N-substituted maleimide derivatives exhibited the same selectivity and a similar activity. These findings point to the same mechanism of their action.

We would express our thanks to the undergraduates GY. GÖNDÖS and T. PROCS for their kind assistance.

Table I.

Zones of inhibition against *Saccharomyces cerevisiae* „GYŐR". The concentration of PMI was 100 p. p. m. before autoclaving.

pH-values during autoclaving	radius of zones in mm produced by autoclaved and thereafter neutralized solutions
2	7
4	5
6	4
9	0
without autoclaving	7

Table II.

Zones of inhibiton (radius in mm) of different N-substituted maleimides of 10^{-3} M concentration.

test organisms	N-phenylmaleimide	N-o-oxyphenylmaleimide	N-m-nitrophenylmaleimide	N-p-acetylmaleimide	N-ethylmaleimide	N-α-naphtylmaleimide
Actinomucor repens	0	0	0	0	0	0
Aspergillus foetidus	11,5	8,0	8,5	7,0	5,9	7,0
Aspergillus niger	12,3	7,0	7,5	8,6	8,5	11,3
Aspergillus oryzae	10,8	9,2	7,5	6,8	9,5	7,3
Circinella minor	0	0	0	0	0	0
Syncephalastrum racemosum	0	0	0	0	0	0

References

FERENCZY L.—ZSOLT J.—MATKOVICS B. (1959) Studies on the antimicrobial activity of N-substituted maleimides. I. Fungistatic activity of N-phenylmaleimide. Acta Biol. Hung., *10*, 77—83.

MATKOVICS B.—FERENCZY L.—SELMECZI GY. (1958) Synthesis of some N-substituted maleic imides. Acta Phys. et Chem. Szeged, *4*, 134—143.

OVERBEEK, J. van — BLONDEAU, R.—HORNE, V. (1955) Maleimides as auxin antagonists. Amer. J. Botan. *42*, 205—213.

VELDSTRA, H. (1946) Enkele grepen uit chemie en physiologia van groei- en remstoffen, Landbouwk. Tydschr. *58*, 489—499.